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Comparative Study of Three PCR Assays With Antigenaemia and Serology for the Diagnosis of HCMV Infection in Thoracic Transplant Recipients

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Three PCR assays were evaluated for the detection of human cytomegalovirus (HCMV) infection in heart and lung transplant recipients in comparison with HCMV antigenaemia and serology assay. Polymorphonuclear leucocyte (PMNL) samples taken at regular intervals after transplantation were tested for HCMV DNA using primer sets homologous to the glycoprotein B (gp58), major immediate early (IE1), and structural phosphoprotein (pp150) regions.

The detection of HCMV infection at various times after transplantation showed all three primer sets to have a sensitivity of 100% and a specificity of 92.3% for the detection of HCMV infection although overall the gp58 primer set was found to be significantly more frequently associated with a positive PCR result than the IE1 ($P = 0.0228$) and pp150 ($P = 0.0015$) primer sets. The positive PCR result had a positive predictive value of 27.8% for HCMV disease. Detection of HCMV infection was first by the PCR assay, and significantly before the HCMV antigenaemia assay.

Of nine patients who received antiviral therapy while PCR positive, only one patient cleared HCMV DNA from PMNLs during treatment but became positive again 17 days later. Quantitative PCR methodologies may improve the predictive value of PCR for HCMV disease and its value for monitoring antiviral therapy.

KEY WORDS: HCMV, PCR, DNA, glycoprotein B, major immediate early region, structural phosphoprotein pp150

INTRODUCTION

HCMV infection in lung and heart transplant recipients is recognised as a major source of morbidity and mortality. The incidence of HCMV infection following thoracic organ transplantation has been shown to vary between 45% and 72% [Smyth et al., 1991; Ettinger et al., 1992; Wreghitt et al., 1988; Egan et al., 1995].

Conventional virological diagnosis by serology and virus isolation only provides a retrospective diagnosis of HCMV infection, but detection of HCMV antigenaemia has been demonstrated to provide an early, rapid diagnosis of HCMV infection [van den Berg et al., 1991; van der Bij et al., 1989; Landry and Ferguson, 1993; Koskinen et al., 1993]. Viral load may be assessed by quantitation of antigenaemia detection, potentially allowing early antiviral therapy to be administered before the onset of clinical symptoms.

The detection of HCMV by DNA amplification techniques (PCR) also provides the potential for rapid early diagnosis [Bitsch et al., 1993; Gerna et al., 1991; Einsele et al., 1991; Wolf and Spector, 1993; Brytting et al., 1992b; Demmler et al., 1988; Storch et al., 1994]. PCR is able to selectively amplify and detect specific DNA sequences. However, sequence variation has been shown to affect performance [Chou, 1992b] and the contribution of different primer sets to the diagnostic sensitivity and specificity of PCR assays therefore requires careful evaluation. Similarly, the development of sensitive DNA based diagnosis requires careful evaluation against other available technologies.

In this study, the efficacy of PCR for the diagnosis and longitudinal detection of HCMV infection was examined in a cohort of 32 thoracic transplant recipients. It was shown previously that 31% of this cohort developed a symptomatic HCMV infection and 15% developed HCMV disease following transplant [Egan et al., 1995]. The performance of three primer pairs homologous to different regions of the HCMV genome were evaluated and the results were compared with conventional diagnostic techniques and HCMV antigenaemia detection.

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MATERIALS AND METHODS

Patients

Between December 1992 and August 1993, 560 sequential EDTA blood samples for detection of HCMV DNA by PCR were obtained from 32 patients undergoing thoracic organ transplantation; 23 patients received cardiac transplants, two patients received heart/lung transplants, two patients received double lung transplants, and five patients received single lung transplants. Samples for PCR were collected during the first week following transplant and then weekly until week 18 (126 days). Sixteen (50%) patients were HCMV IgG antibody positive before transplantation, the other 16 were HCMV IgG antibody negative pre-transplant. HCMV seronegative recipients of organs from seropositive donors received prophylactic intravenous HCMV hyperimmune globulin on days 7, 14, 21, 28, and 49 (Alpha Therapeutic UK, Thetford, Norfolk).

Antiviral Therapy

Patients received ganciclovir when clinical symptoms potentially due to HCMV were diagnosed. Ganciclovir was administered at 5 mg/kg every 12 hr for 21 days in the presence of HCMV disease or at the discretion of the clinician for symptomatic patients.

Routine HCMV Surveillance and Antigenaemia Detection

The HCMV antibody status of the organ donor and recipient was determined prior to transplant (CMV scan; Becton Dickinson). Routine post-operative surveillance involved routine virus culture from urine and throat swabs, detection of specific IgG and IgM antibody by enzyme immunoassay [Morris et al., 1990], and examination of bronchoalveolar lavage (BAL) specimens for the detection of early antigen fluorescent foci [Morris et al., 1987]. The detection of pp65 antigen in polymorphonuclear leucocytes (PMNL) was as described previously [Egan et al., 1995]. Routine surveillance samples were collected on the first post-operative day and then weekly for 6 weeks, two-weekly until week 12, and monthly thereafter.

HCMV DNA Detection by PCR

a) Extraction of nucleic acid. PMNL samples were tested for HCMV DNA by PCR. Total nucleic acid was extracted from PMNLs by resuspending 2×10^6 cells in 200 μl of the proteinase K buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.5% Nonidet P40, 0.5% Tween 20, proteinase K 100 μg/ml) and incubated for 1 hr at 55°C. The nucleic acid was extracted once with phenol-chloroform, precipitated with ethanol, and resuspended in 200 μl of sterile injectable water.

b) Oligonucleotides. PCR for the detection of HCMV DNA was carried out using oligonucleotide primers described previously and PCR products were probed with a complementary 5′digoxigenin-labelled internal oligonucleotide probe [Zipeto et al., 1990; Darlington et al., 1991] (Table I). The primers chosen amplified in the glycoprotein B (gp58), major immediate early (IE1), and the structural matrix phosphoprotein (pp150) regions of the HCMV genome.

c) PCR assay. Reaction conditions were optimised for each set of primers. The reaction mixture consisted of 25 mM Tris-HCl pH 8.4, 17 mM $(NH_4)_2SO_4$, 0.5 mM β-mercaptoethanol, 0.1% gelatin, deoxynucleoside triphosphates (200 μM each), 2.5 units of *Taq* DNA polymerase (Gibco, BRL), 2 mM $MgCl_2$, two primers (1 μM each), and 20 μl of template DNA (see below).

Amplification of the gp58 and IE1 gene region involved denaturation for 4 min at 94°C followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, followed by a final incubation at 72°C for 7 min. Thermal cycling conditions for the primer set to the pp150 gene region were as above except annealing was carried out at 55°C.

Water and DNA extracted from healthy HCMV seropositive and seronegative individuals were included as negative controls.

PCR products were examined on a 3% 3:1 Nusieve agarose gel (FMC, Flowgen) and Southern blotted onto nylon membrane (Boehringer Mannheim, Germany) by capillary blotting [Sambrook et al., 1989]. Transferred DNA was cross-linked to the nylon membrane by UV irradiation, prehybridised for 1 hr in a standard hybridisation solution (5 × SSC, 0.1% sodium lauroylsarcosine, 1% blocking reagent [w/v] [Boehringer Mannheim, Germany], 0.02% SDS), and hybridised overnight with the appropriate 5′digoxigenin-labelled oligonucleotide probe (20 ng/ml) in the standard hybridisation solution at 42°C. Filters were then washed for 2 × 5 min in 2 × SSC, 0.1% SDS at room temperature and 2 × 15 min in 1 × SSC, 0.1% SDS at 55°C. Hybridised probes were detected by chemiluminescence, according to the manufacturers' protocol (Boehringer Mannheim, Germany).

To evaluate negative results due to the presence of PCR inhibitors or failed DNA extractions, samples were amplified using primers to the human β-globin gene region [Bauer et al., 1991] which gave a 268 bp PCR product and were examined on a 3% 3:1 Nusieve agarose gel.

Clinical Definitions

The clinical outcome of HCMV infection was defined as asymptomatic infection, symptomatic infection or disease using the following criteria:

1. Asymptomatic HCMV infection was defined as a positive culture for HCMV from a non-peripheral site and/or a positive IgM antibody response (>3 arbitrary units) and/or a positive antigenaemia detection;
2. Symptomatic HCMV infection was defined as an unexplained pyrexia with or without a 50% fall in the white cell count and/or a 2.5-fold increase in serum transaminases in the presence of a positive IgM response (>3 arbitrary units) and/or positive

TABLE I. Sequences of Oligonucleotide Primers and Probes Used for the Amplification and Detection of HCMV Genome

Gene region	Primer pair	Probe	Product length, bp
gp58	P1 5'GAGGACAACGAAATCCTGTTGGGGA'3 P2 5'TCGACGGTGGAGATACTGCTGAGG'3	5'CCCAGCCTCAAGATCTTCAT'3	149
IE1	P1 5'AGACCTTCATGCAGATCTCC'3 P2 5'GGTGCTCACGCACATTGATC'3	5'CTAGTGTGATGCTGGCCAAGCGGCCTCTGA'3	263
pp150	P1 5'ATCCCTTCAGGATGCCTACG'3 P2 5'GACGTCGTTGTTGTAATCGG'3	5'GACCAAACTGCAGAGTCACC'3	257

BAL DEAFF test and/or positive antigenaemia detection;

3. HCMV disease was defined as histological evidence of end organ damage.

Statistical Analysis

The Wilcoxon signed ranks test was used to analyse the time to diagnosis of an active HCMV infection by the PCR assay, compared with antigenaemia detection and onset of clinical symptoms. The Mann Whitney U test was used to analyse differences between patients who developed symptomatic HCMV infection and HCMV disease. The percentage of positive samples associated with each oligonucleotide primer set were also analysed using the Mann Whitney U test. Confidence intervals were set at 95% and exact probability P values given are two-tailed. Sensitivity, specificity, and predictive values were calculated as described previously [Vecchio, 1966].

RESULTS

HCMV infection was diagnosed in 17 of a total of 32 patients using standard methodologies and/or HCMV antigenaemia detection (one patient was asymptomatic, 11 were symptomatic, five developed HCMV disease). The remaining 15 patients had no laboratory evidence of infection using standard methology and were free of clinical symptoms.

PCR Results

a) Evaluation of primers and probes to detect positive samples. All blood samples (n = 560) taken longitudinally following transplant were amplified with each set of primers. Overall, the gp58 primer set was significantly more frequently associated with a positive result than the IE1 (p = 0.0228) and pp150 (p = 0.0015) primer sets. All amplified products visualised directly on an ethidium bromide-stained agarose gel were detected on the Southern blot. Probing allowed an increase in the sensitivity of detection for all three primer sets. The number of positive results detected by direct gel analysis was greatest using the gp58 primer set (Table II).

b) Sensitivity and specificity. Detection of HCMV by PCR showed 18 patients to be HCMV DNA positive including all 17 patients with conventionally diagnosed HCMV infection (Table III). All 12 transplant patients with recipient negative/donor negative HCMV antibody status and two patients with recipient negative/donor positive HCMV antibody status remained PCR negative.

Each primer set showed a sensitivity of 100% for the detection of HCMV infection. Of 15 patients with no evidence of an active HCMV infection by conventional methods, one patient (patient 18) was PCR positive with all three primer sets. This patient, who was HCMV seropositive prior to transplant and received a double lung transplant from an HCMV seronegative donor, showed no clinical symptoms of an HCMV infection.

In accordance with the criteria established for diagnosis of an active HCMV infection, all three primer sets had a specificity of 92.3%. Antigenaemia detection had a sensitivity of 100% and a specificity of 93.7% for HCMV infection. The specificity of PCR for detecting patients who developed HCMV disease was lower than that for detection of HCMV infection. Each primer set gave a specificity of 48% and a sensitivity of 100% for HCMV disease.

c) Predictive values for infection and disease. For each primer set the positive predictive value for HCMV infection was 94.4% compared to 94.1% for antigenaemia detection. The positive predictive value of HCMV disease associated with the detection of HCMV DNA was 27.8% compared to 45.5% for antigenaemia levels greater than 50 positive PMNLs/2 $\times$ 10^5 PMNLs. The negative predictive value of HCMV DNA detection for HCMV infection and disease was 100% for each primer set.

d) First diagnosis of HCMV infection by the PCR assay: Comparison of three primer sets. The gp58 primer set first became positive at a median of 7.5 days (range 1–42) following transplant. The IE1 primer set and pp150 primer set first became positive at a median of 14.5 days (range 1–81) and 13 days (range 1–42), respectively. Thee differences were found not to be of significance (P = 0.2070 gp58 and IE1, P = 0.1250 gp58 and pp150, P = 0.8124 IE1 and pp150) (Table IV).

The median number of days to first positive PCR result following transplant for patients who died within 126 days post-transplant (n = 3) was 16 days (range 7–42) for each set of primers. The median number of days to first PCR result following transplant for patients who survived (n = 15) was 6 days (range 1–27) for the gp58 primer set, 10 days (range 1–81) for the IE1 primer set, and 13 days (range 1–31) for the pp150 primer set. The differences in time to detection by each of the primer

TABLE II. Comparison of Three Primer Sets for the Detection of Positive Samples by PCR*

Primer set	% and number of samples positive following detection by Southern blotting (n = 560)	% and number of positive samples detected by direct gel analysis
gp58	51% (n = 288)	91% (n = 262)
IE1	39% (n = 219)	55% (n = 121)
pp150	40% (n = 226)	40% (n = 91)

*The positive PCR result was significantly more frequently associated with the gp58 oligonucleotide primer set ($P = 0.0228$ IE1, $P = 0.0015$ pp150).

TABLE III. Laboratory Detection of HCMV Infection and Clinical Symptoms in Thoracic Organ Transplant Recipients*

Patient	Organ transplant	Pretransplant serology		Symptomatic infection	HCMV DNA PCR			pp65-antigen positive PMNLs/2 × 10^5 PMNLs	IgM[a]
		Donor	Recipient		gp58	IE1	pp150		
1	H	+	+	Yes	+(3)	+(3)	+(3)	368 (33)	−
2	H	+	+	No	+(7)	+(7)	+(7)	3(59)	−
3	H	+	−	Yes	+(13)	+(81)	+(13)	55(61)	−
4	H	+	+	Yes	+(4)	+(4)	+(4)	18(19)	+(33)
5	H	+	+	Yes	+(15)	+(15)	+(15)	53(21)	+(28)
6	H	−	+	Yes	+(4)	+(18)	+(18)	120(18)	+(60)
7	H	−	−	Yes	+(1)	+(1)	+(1)	558(93)	+(121)
8	H	−	+	Yes	+(13)	+(13)	+(13)	1386(13)	+(80)
9	H	−	+	Yes	+(16)	+(8)	+(16)	2916)	+(69)
10	HL	−	+	Yes	+(4)	+(4)	+(18)	1(58)	+(49)
11	SL	−	+	Yes	+(6)	+(6)	+(13)	2(34)	−
12	SL	+	+	Yes	+(8)	+(14)	+(31)	1(35)	−
13	SL	+	+	Yes[b]	+(4)	+(34)	+(4)	73(65)	+(118)
14	SL	−	+	Yes[b]	+(11)	+(38)	+(11)	200(50)	+(93)
15	H	+	+	Yes[b]	+(16)	+(16)	+(16)	152(29)	+(36)
16	H	+	+	Yes[b]	+(27)	+(27)	+(27)	2647(50)	+(64)
17	H	+	−	Yes[b]	+(42)	+(42)	+(42)	1200(48)	+(41)
18	DL	−	+	No	+(9)	+(16)	+(9)	−	−

*H, Heart transplant; HL, Heart and lung transplant; SL, Single lung transplant; DL, Double lung transplant. Figures in brackets are number of days post-transplant to first positive result.
[a]>3 arbitrary units antibody response.
[b]These patients developed HCMV disease.

TABLE IV. Detection of HCMV Infection Post-Transplant by PCR, Antigenaemia, and Serology*

	PCR			Antigenaemia	IgM	Clinical symptoms
	gp58	IE1	pp150			
Median days first positive post-transplant	7.5	12	13.5	35	64	65

*Detection of HCMV infection by PCR was positive significantly before the antigenaemia assay ($P = <0.0001$ gp58, $P = 0.0009$ IE1, $P = 0.0001$ pp150).

sets in those patients who survived and those who died were not found to be of significance ($P = 0.1275$ gp58, $P = 0.3848$ IE1, $P = 0.4240$ pp150).

e) Comparison of the PCR assay with serology and antigenaemia assay for the early diagnosis of HCMV infection. A clinical diagnosis of HCMV related illness was made at a median of 65 days (range 19–142), serological detection of an IgM response occurred at a median of 64 days (range 28–267), and antigenaemia first became positive at a median of 35 days (range 13–93) following transplant (Table IV). PCR detection of HCMV infection occurred significantly before detection of antigenaemia for all three sets of primers ($P = <0.0001$ gp58, $P = 0.0009$ IE1, $P = 0.0001$ pp150) and remained positive longer than antigenaemia detection. PCR detection first became positive at a median of 28 days (range 0–93) for the gp58 primer set, 21 days (range 0–93) for the IE1 primer set, and 22 days (range 0–93) for the pp150 primer set before the onset of first antigenaemia detection.

Patient 3 was seen to be PCR positive with the gp58 and pp150 primer sets 48 days before first antigenaemia detection but was first positive with the IE1 primer set 20 days after the first antigenaemia result. In the case

of patient 13, the IE1 primer set first became positive 30 days after the gp58 and pp150 primer sets were first positive. Both these patients received donor organs from the same patient (Table III).

f) Comparison of different primer sets for the detection of HCMV infection before the onset of clinical symptoms. Detection of HCMV by PCR in patients with a symptomatic infection occurred at a median of 52 days (range 10–142) before the onset of clinical symptoms for the gp58 and pp150 (range 10–142) primer sets and at a median of 43 days (range 8–142) for the IE1 primer set. In patients who developed HCMV disease, each primer set was first positive at a median of 42 days (range 37–72 for the gp58 and pp150 primer sets, range 37–45 for the IE1 primer set) before the onset of clinical symptoms. These differences were found not to be of significance ($P = 0.4618$ gp58 and IE1, $P = 0.1250$ gp58 and pp150, $P = 0.3096$ IE1 and pp150). Clinical symptoms occurred three days before a positive PCR result in patient 17. Samples from this patient had not been collected 3 weeks prior to clinical symptoms.

The differences in days to first positive PCR results before the onset of clinical symptoms for the different primer sets were not shown to be significantly different between symptomatic patients and those who developed HCMV disease ($P = 0.4913$ gp58, $P = 0.4396$ IE1, $P = 0.6424$ pp150).

Monitoring of Antiviral Therapy

Of 10 patients who received ganciclovir, seven patients were treated while antigenaemia positive and three received treatment after antigenaemia had already become negative.

Following ganciclovir therapy all patients became antigenaemia negative (Fig. 1). Antigenaemia levels in patient 7 became highest after 126 days post-transplant and remained positive until the patient died on day 241. This patient received augmented immunosuppression for life threatening cardiac rejection and was given two courses of ganciclovir therapy but remained antigenaemia and PCR positive.

The gp58 primer set was used in the PCR assay to detect HCMV DNA in PMNLs during ganciclovir treatment as this primer set was significantly associated with a positive result. PCR detection of HCMV DNA following ganciclovir was not followed up in patient 3 after receiving ganciclovir. Of the remaining nine patients who were treated with ganciclovir, eight patients remained PCR positive during ganciclovir therapy and one patient (patient 5) cleared HCMV DNA from the blood. Two of the patients who were PCR positive during ganciclovir therapy died during treatment (patients 2 and 17) and the others remained PCR positive following treatment. Patient 5, who cleared HCMV DNA, became positive again 17 days later.

Four of the 10 patients treated with ganciclovir developed HCMV disease, five patients developed symptomatic infection, and one patient remained asymptomatic. This last patient (patient 2) died on day 110 following transplant, having begun ganciclovir therapy on day 105. Two of the patients who developed disease died; patient 17 died on day 57 due to graft failure, pancreatitis, and HCMV duodenitis; patient 15 died on day 58 from acute cholecystitis and septicaemia. All symptomatic patients survived more than 126 days.

DISCUSSION

In a previous study [Egan et al., 1995] it was demonstrated that monitoring for HCMV pp65 antigenaemia following heart and lung transplant detects active HCMV infection before the onset of clinical symptoms and identifies patients at risk of developing HCMV disease. This study showed that qualitative detection of HCMV DNA by PCR has a high degree of sensitivity and specificity for the detection of asymptomatic and symptomatic HCMV infections but a lower specificity for those patients who developed HCMV disease, and was only associated with a relative risk of 27.8% for the disease.

The comparison of different primer sets in PCR assays showed a positive PCR result to be significantly more frequently associated with the glycoprotein B (gp58) primer set than with the major immediate early (IE1) and structural matrix phosphoprotein (pp150) primer sets. Differences were also seen between the time to detection of HCMV infection by PCR following transplant and the onset of clinical symptoms among the different primer sets, although they were not significant.

Only 55 and 40% of positive samples amplified by the IE1 and pp150 primer sets, respectively, compared to 91% of positive samples amplified by the gp58 primer set could be visualised directly by ethidium bromide staining. Other diagnostic PCR assays also based upon the immediate early region have been used for the detection of HCMV in clinical samples [Gerna et al., 1991; Demmler et al., 1988; Einsele et al., 1991; Jiwa et al., 1989]. This region of the HCMV genome has been shown to possess sporadic sequence variation among different clinical strains [Chou, 1992; Brytting et al., 1992] and primer mismatching has been shown to reduce amplification efficiency of a PCR assay [Chou, 1992]. In contrast, although sequence variation also exists within the gp58 gene region among different HCMV clinical strains, these variations are highly clustered [Chou, 1992]. The glycoprotein B primer sequences used in this study were homologous to the non-clustered regions of the glycoprotein B gene region and were greater in length than primer sequences to the major immediate early and structural phosphoprotein gene regions. Optimal primer design and greater primer length may account for the increase in sensitivity of the glycoprotein B primer pair. The effect of a mismatched base in longer primers may be lessened and allow for a sensitive PCR assay.

Since PCR is a very sensitive technique for the detection of DNA, latent virus may be detected in seropositive patients. Discrepant results were seen in one patient who was PCR positive with all three sets of patients but antigenaemia negative and who showed no clinical symptoms of an active HCMV infection. However, HCMV DNA was not detected in PMNL samples from

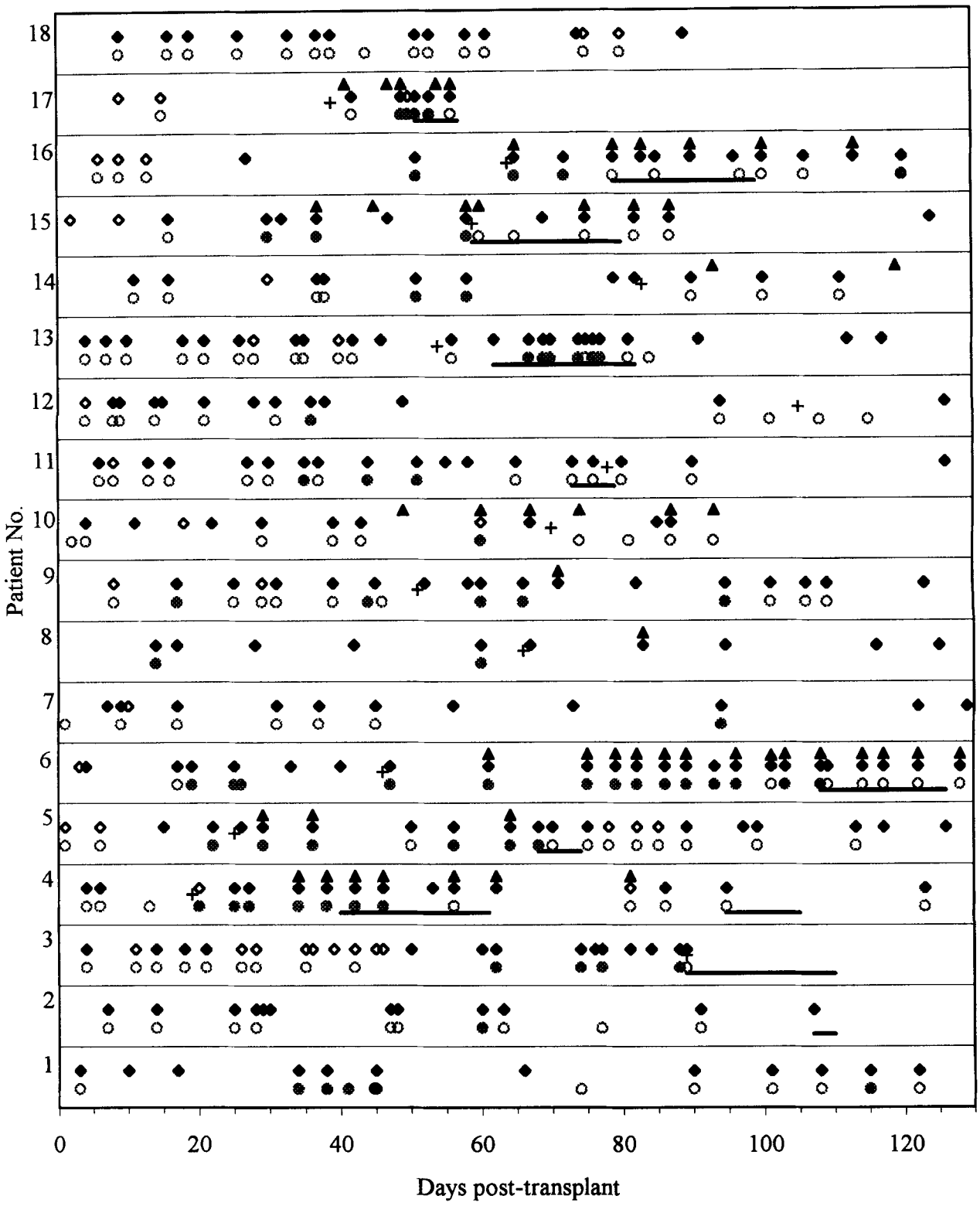

Fig. 1. Schematic representation of the detection of HCMV DNA in PMNLs, antigenaemia, serology, clinical symptoms, and antiviral therapy in 18 heart, heart/lung, and lung transplant recipients post-transplant. Patients 3, 10, and 18 were followed up for only 89 days post-transplant and patients 2, 15, and 17 died when positive for HCMV DNA and before 126 days post-transplant. ♦ HCMV DNA positive ◇ HCMV DNA negative ● Antigenaemia positive ○ Antigenamia negative ▲ Detection of IgM + Clinical symptoms —— Ganciclovir therapy

healthy HCMV seropositive individuals tested by PCR, and patients HCMV seropositive prior to transplant were PCR positive after initial PMNL samples were PCR negative. These findings are consistent with the view that PMNLs do not harbour latent virus and that detection of HCMV DNA in PMNLs is associated with an active HCMV infection [Taylor-Wiedeman et al., 1993].

The detection of HCMV infection by PCR became positive significantly earlier than detection of antigenaemia and remained positive longer. No significant difference was seen between symptomatic patients and those who developed disease in time to detection of the first positive PCR before the onset of clinical symptoms. The qualitative detection of HCMV DNA in PMNLs by PCR does not appear to be a useful marker for HCMV disease although it may indicate a risk of reactivation of the viral infection in blood. The antigenaemia assay became negative during ganciclovir treatment in all patients. However, HCMV DNA in PMNLs persisted after ganciclovir treatment in both symptomatic patients and those patients who developed HCMV disease, even though all patients improved clinically following ganciclovir therapy. The clinical significance of persistent DNA may vary depending on underlying circumstances, such as graft rejection or the presence of other infectious agents.

In conclusion, qualitative detection of HCMV DNA in PMNLs by PCR does not appear to be a useful marker for HCMV disease in comparison to the antigenaemia assay but quantitative PCR may eventually provide a more accurate indication of clearance of a HCMV infection from the blood after antiviral therapy [Einsele et al., 1991]. Quantitative PCR techniques have been developed and applied to the detection of HCMV [Fox et al., 1992; Schäfer et al., 1993; Fox et al., 1995). Assessment of viral load following organ transplants using quantitative PCR methodologies may provide further information on the predictive value of PCR for disease.

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